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High Affinity Interaction of Syntaxin and SNAP-25 on the Plasma Membrane Is Abolished by Botulinum Toxin E*

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The release of hormones and neurotransmitters requires the fusion of cargo-containing vesicles with the plasma membrane. This process of exocytosis relies on three SNARE proteins, namely syntaxin and SNAP-25 on the target plasma membrane and synaptobrevin on the vesicular membrane. In this study we examined the molecular assembly pathway that leads to formation of the fusogenic SNARE complex. We now show that the plasma membrane syntaxin and SNAP-25 interact with high affinity and equimolar stoichiometry to form a stable dimer on the pathway to the ternary SNARE complex. In bovine chromaffin cells, syntaxin and SNAP-25 colocalize in defined clusters that average 700 nm in diameter and cover 10% of the plasma membrane. Removal of the C terminus of SNAP-25 by botulinum neurotoxin E, a known neuroparalytic agent, dissociates the target SNARE dimer *in vitro* and disrupts the SNARE clustering *in vivo*. Together, our data uncover formation of stable syntaxin/SNAP-25 dimers as a central principle of the SNARE assembly pathway underlying regulated exocytosis.

Hormonal and neurotransmitter release occurs when vesicles fuse with the plasma membrane in a calcium-dependent manner. The three SNARE[1] proteins, namely synaptobrevin2 (also known as VAMP2) on the vesicular membrane and syntaxin1 and SNAP-25 on the target plasma membrane, are essential for the last step of vesicular exocytosis, the fusion of membranes (1, 2). These three proteins form a highly stable ternary complex that likely drives the fusion of the two apposing membranes (3). Botulinum toxins, which specifically cleave SNARE proteins prior to the formation of the ternary SNARE complex, block vesicle exocytosis in both neurons and endocrine cells (2, 4). The plasma membrane protein SNAP-25 is a molecular target for botulinum toxin E (5). This toxin removes the C-terminal 26 amino acids from the SNAP-25 molecule and has been used previously to dissect stages of calcium-triggered exocytosis (6, 7). Although these studies demonstrated that the ternary SNARE complex forms at a late stage of vesicle exocytosis, the molecular events preceding formation of this complex remain largely unknown.

Several contradicting theories have been put forward with regard to the molecular pathway leading to SNARE complex assembly. One study suggested that SNAREs, although active, do not pre-assemble in resting cells into any intermediate complex but would form the ternary complex upon activation of exocytosis (8). It has also been proposed that SNAP-25 engages vesicular synaptobrevin in a priming step and that binding of syntaxin1 would be the pivotal event leading to membrane fusion (9). An alternative theory states that syntaxin1 and SNAP-25 pre-assemble into a stable heterodimer on the plasma membrane, and only then does vesicular synaptobrevin engage the two target SNAREs (10, 11). Importantly, it is not clear whether any SNARE intermediates actually exist in intact secretory cells as a prelude to SNARE-mediated fusion.

To address these outstanding issues, we analyzed SNARE binary reactions in a quantitative manner using highly purified SNARE proteins. We also investigated the distribution of the three SNARE proteins in chromaffin cells, a well established model system for regulated secretion (12, 13). Analysis of the SNARE assembly pathway reveals that the only stable heterodimer is composed of the two target SNAREs, syntaxin1 and SNAP-25. These two proteins not only interact with high affinity in an equimolar stoichiometry but also colocalize in defined clusters on the plasma membrane of chromaffin cells. The absence of synaptobrevin in the syntaxin1/SNAP-25 clusters indicates that this vesicular SNARE does not contribute to the cluster stability in chromaffin cells. Finally, we show that botulinum toxin E disrupts the syntaxin1/SNAP-25 heterodimers both *in vitro* and *in vivo*, demonstrating that they can be molecular targets of botulinum toxin poisoning.

EXPERIMENTAL PROCEDURES

Preparation of Proteins—Plasmids encoding glutathione *S*-transferase (GST) fusion proteins with syntaxin (amino acids 1–261) and synaptobrevin2 (amino acids 1–96) were described previously (10). Recombinant SNARE proteins, purified on glutathione-Sepharose beads (Amersham Biosciences), were washed with buffer A (20 mM HEPES, pH 7.0, 100 mM NaCl, 2 mM EDTA) followed by elution with 15 mM reduced glutathione in buffer A or thrombin cleavage. Eluted proteins were further purified by gel filtration on a Superdex 200 column (Amersham Biosciences) equilibrated in buffer A. SNAP-25 and native SNARE complex was purified from bovine brain cortex as described previously (10). For constructing the expression plasmid for the light chain of botulinum toxin E, the DNA fragment encoding the light chain was generated by PCR using total bacterial DNA (strain Beluga) as a template and 5′-CACAGGATCCATGCCAAAAATTAATAGTTTTAA-3′ and 5′-CTCTCAAGCTTAGGCCTTATGCCTTTTACAGAAAC-3′ as forward and reverse primers, respectively. The fragment was inserted subsequent to cleavage with BamHI and HindIII into pQE9 (Qiagen). Thereafter, the self-complementary oligonucleotide 5′-TAAGCTAATTTAAATTAGCTTA-3′ was ligated into the singular StuI site to introduce

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[1] The abbreviations used are: SNARE, soluble NSF attachment protein receptor; NSF, *N*-ethylmaleimide-sensitive factor; SNAP, soluble NSF attachment protein; GST, glutathione *S*-transferase; PIPES, 1,4-piperazinediethanesulfonic acid; BoNT/E, botulinum toxin E; LC, light chain.

the translation termination codon. The nucleotide sequence was verified by DNA sequencing. The His_6-botulinum toxin E light chain was expressed at 21 °C for 16 h. Protein was purified on a HiTrap nickel chelating column (Amersham Biosciences) and washed with 20 mM imidazole in buffer B (20 mM HEPES, pH 7.0, 100 mM NaCl) followed by elution with 100 mM imidazole in buffer B. Imidazole was removed by dialysis against buffer B.

Quantitative Protein Binding Assays—SNARE binding reactions were performed at room temperature for 30 min. Two-microgram SNARE proteins attached to either glutathione or anti-SNAP-25 (SMI81)-Sepharose beads (~15-μl bed volume) were incubated in buffer A with 3 μg of protein to be tested for binding. Beads were washed three times by low speed centrifugation with 1 ml of buffer A, and bound protein was eluted in SDS-containing sample buffer followed by SDS-PAGE and Coomassie staining. For analysis of the effect of the botulinum toxin E light chain on SNAP-25 in the preformed syntaxin1/SNAP-25 heterodimer, the binary complex immobilized on Sepharose beads was incubated with a purified botulinum toxin E light chain for 1 h at 37 °C. The beads were then washed three times with 1 ml of buffer A before analysis of bound protein by SDS-PAGE and Coomassie staining. To determine the dissociation constant for the syntaxin1/SNAP-25 heterodimer, anti-SNAP-25-Sepharose beads containing 1 pmol of SNAP-25 were incubated for 2 h with increasing concentrations of syntaxin-(1–261) (from 10^{-9} to 10^{-5} M) in a reaction volume of 100 μl. The beads were washed three times with 1 ml of buffer A, and bound protein was analyzed by Western immunoblotting using a rabbit polyclonal anti-syntaxin1 antibody and a West Dura enhanced chemiluminescence kit (Pierce). The chemiluminescence signal was imaged using a ChemiDoc XRS (Bio-Rad) and quantified using Quantity One software (Bio-Rad). The calculated intensity volumes were fitted with a variable slope dose-response relationship using Prism (GraphPad). For determination of the stoichiometry of SNAREs in the syntaxin1/SNAP-25 heterodimer, proteins bound to the beads were eluted into sample buffer and separated by SDS-PAGE. Gels were stained with Sypro Orange (Bio-Rad) according to the manufacturer's instructions. Fluorescent protein bands were imaged using a ChemiDoc XRS, (Bio-Rad), and fluorescent intensity was quantified using Quantity One software (Bio-Rad). To calculate the molar ratio of interacting proteins, the measured values of bound fluorescence for each band were divided by their corresponding molecular masses.

Confocal Microscopy—Bovine chromaffin cells seeded on polylysine-coated bottom glass coverslip dishes (Mattek) were washed, fixed with 4% paraformaldehyde for 20 min, and then blocked for 1 h in phosphate-buffered saline containing 3% goat serum and 0.05% Triton X-100. Rabbit anti-SNAP-25 polyclonal antibody (1:1000 dilution) and mouse anti-syntaxin1 antibody (HPC-1 clone, Sigma; 1:1000) were added, and cells were incubated overnight at 4 °C. In some cases, chromaffin cells were permeabilized with 0.002% digitonin in 139 mM potassium glutamate, 20 mM PIPES, 5 mM EGTA, 2 mM ATP, and 2 mM $MgCl_2$, pH 6.5 in the absence or presence of botulinum toxin E light chain (5.4 μg/ml) for 15 min prior to fixation. Cells were washed extensively and stained using Alexa 488 anti-rabbit and Alexa 594 anti-mouse (Molecular Probes) for 1 h. Cells were washed overnight in phosphate-buffered saline. Images were collected on a Bio-Rad 600 confocal microscope using an oil immersion objective (63×; 1.4 numerical aperture). The point spread function of green and red fluorescence was calculated as described (14). Fluorescence intensity was quantified using the LaserPix software (Bio-Rad). Areas of the target SNAREs were estimated using Equation 1,

$$A_{(\text{SNAP-25/syntaxin1})} = ((A_{(\text{SNAP-25})} \times \%FC) + 0.5*(A_{(\text{SNAP-25})} \times \%NC)) \times 100/A_{(\text{TotChr})} \quad \text{(Eq. 1)}$$

where $A_{(\text{SNAP-25/syntaxin1})}$ is the area occupied by SNAP-25 clusters that colocalize with syntaxin1, $A_{(\text{SNAP-25})}$ is the area occupied by SNAP-25 clusters, *%FC* is the percentage of full colocalization between SNAP-25 and syntaxin1, *%NC* is percentage of neutral colocalization between SNAP-25 and syntaxin1, and $A_{(\text{TotChr})}$ is the average total area of the plasma membrane of chromaffin cells estimated to be 801.6 μm^2 (n = 120).

RESULTS

Exocytosis from neuroendocrine cells relies on syntaxin1, SNAP-25, and synaptobrevin2 (1, 13, 15). To determine the spatial organization of the SNARE fusion machinery, we chose to study chromaffin cells, which have a simple round geometry and serve as a popular model of regulated exocytosis. We used antibodies that recognize the SNARE proteins even when in complexes (data not shown) in conjunction with high resolution confocal microscopy. Chromaffin cells isolated from bovine adrenal glands were fixed with 4% paraformaldehyde and immunostained for the two target SNAREs. Syntaxin1 and SNAP-25 clearly localized to the plasma membrane and, significantly, showed a high degree of colocalization within spots of high intensity in equatorial confocal sections (Fig. 1*A*). To investigate the distribution of syntaxin1 and SNAP-25 in the plane of the plasma membrane, polar confocal sections of chromaffin cells were also studied. Remarkably, the distribution of the two target SNAREs on the plasma membrane showed an extremely high degree of colocalization, with the two proteins co-existing in defined clusters (Fig. 1*B*).

Using the high resolution polar confocal sections, we quantified the degree of syntaxin1/SNAP-25 colocalization and measured their area and surface density (Fig. 2*A*). Cluster analysis used previously for PC12 cell membrane sheets (14) demonstrated that, in chromaffin cells, syntaxin1 and SNAP-25 are highly colocalized in clusters (Fig. 2*B*). Ninety percent of the syntaxin1 and SNAP-25 immunoreactivity exhibited perfect overlap within these clusters, which averaged ~700 nm in diameter and 0.43 μm^2 by area (Fig. 2*B*). A frequency histogram of SNARE cluster area (Fig. 2*C*) highlights the matching distribution for the two proteins, suggesting that they interact on the plasma membrane. In total, the SNARE clusters occupy ~10% of the total chromaffin cell plasma membrane.

We next investigated whether the vesicular SNARE synaptobrevin plays a role in the organization of the two target SNAREs on the plasma membrane. Using double immunostaining for SNAP-25 and synaptobrevin on both equatorial and polar confocal sections, we found that the majority of synaptobrevin immunoreactivity resides in vesicles away from the plasma membrane (Fig. 3*A*). In the polar sections, SNAP-25 clusters were devoid of synaptobrevin immunoreactivity, indicating that the stable target SNARE clustering does not require the vesicular SNARE. Because staining for two more vesicular markers, deoxy-β-hydrolyze (Fig. 3*B*, *DBH*) and synaptotagmin1 (Fig. 3*B*, *Syt*), demonstrates segregation of vesicular and plasma membrane immunofluorescence (Fig. 3*B*), colocalization of the target SNAREs on the plasma membrane is likely irrespective of vesicular proteins. This was predictable, because previous electron microscopy studies demonstrated that the vast majority of secretory granules reside away from the plasma membrane in the resting cells (16).

The strict colocalization of syntaxin1 and SNAP-25 in chromaffin cells suggests that they may interact on the plasma membrane. To address the current conflicting hypotheses of protein interactions in the SNARE assembly pathway, we purified full-length SNAP-25 from the brain cortex and isolated the recombinant cytoplasmic parts of syntaxin1 and synaptobrevin2. We chose to use the SNAREs without their transmembrane regions (TMRs) because TMR-TMR interactions, according to the SNARE paradigm, follow interactions between the cytoplasmic SNARE parts and only occur upon membrane fusion. To assess the dimeric interactions between the highly purified SNAREs, we used Coomassie protein gel staining rather than Western immunoblotting, because the former provides an immediate estimate of binding efficiency and does not exaggerate weak, low affinity interactions, as can be the case for immunoblotting. Individual SNARE proteins were immobilized on Sepharose beads and incubated with the other cognate SNAREs, and bound protein was analyzed after extensive

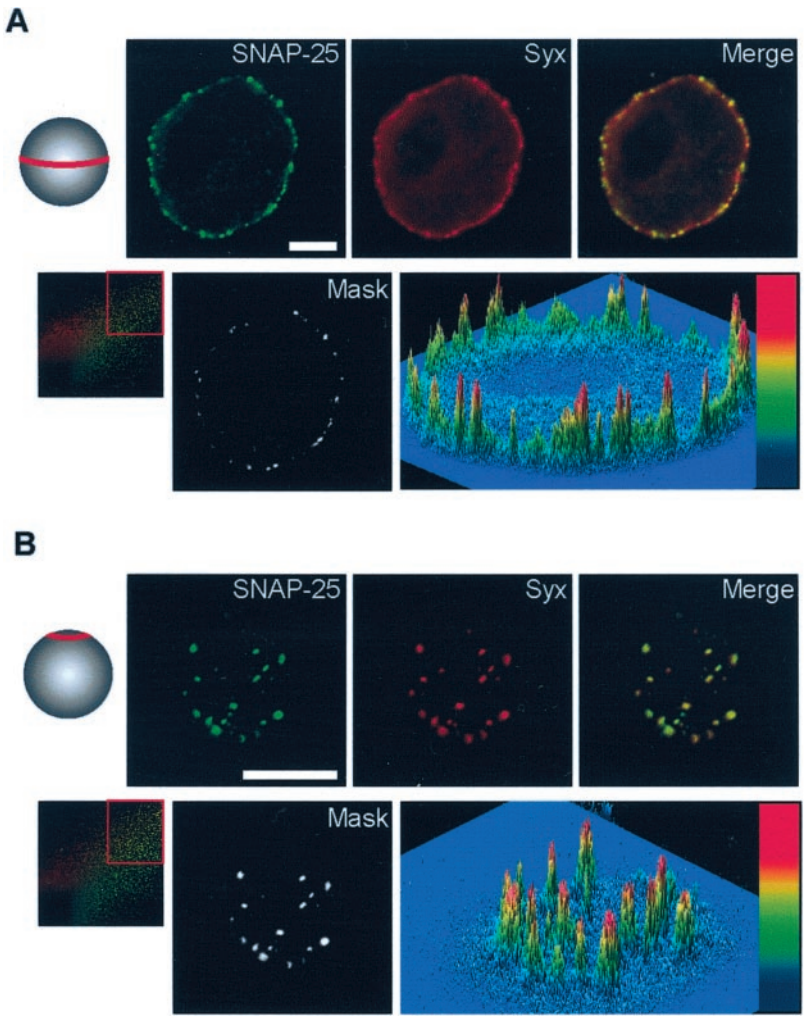


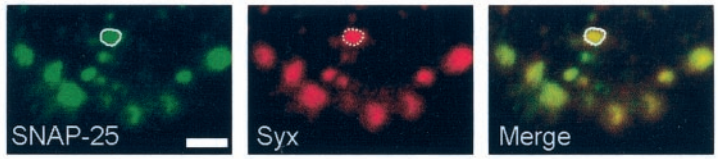
FIG. 1. **The chromaffin cell plasma membrane contains defined patches of syntaxin1 (*Syx*) and SNAP-25.** *A*, equatorial optical confocal section of fixed chromaffin cell immunostained for SNAP-25 and syntaxin1 as indicated. Both markers are concentrated on the plasma membrane, and areas of strong colocalization can be observed in the merge channel. The scatter diagram of the pixel intensity distribution from this confocal section is shown on the *left*. Colocalized pixels (>100 arbitrary units, *red box*) are shown in the mask image (in *white*), indicating co-existence of high intensity spots on the plasma membrane. Surface plot of the merge panel shows, on a color-coding scale (*red* = high), that the relative intensities of the colocalized spots are highly enriched on the plasma membrane. *B*, polar optical confocal section of the above chromaffin cell revealing clusters of high immunoreactivity for both SNAP-25 and syntaxin1 on the plasma membrane. These clusters exhibit a high degree of colocalization as shown in the merged image. The scatter diagram of the pixel intensity distribution from this confocal section is shown on the *left*. High intensity colocalization (>100 arbitrary units) can be seen in the mask image. Surface plot of the merge panel indicates, on the color-coding scale, the relative intensity of the colocalized clusters. *Scale bars* in all images represent 5 μm.

washing of the beads. The addition of SNAP-25 and synaptobrevin to immobilized syntaxin1 resulted in the binding of SNAP-25 only (Fig. 4*A*). Immobilized SNAP-25, when incubated with its two partners, bound only to syntaxin1 and not to synaptobrevin (Fig. 4*B*). Finally, immobilized synaptobrevin did not interact with either of the monomeric target SNAREs alone but bound them both when incubated together (Fig. 4*C*). These experiments indicate that syntaxin1 and SNAP-25 *in vitro* form a dimeric precursor on the pathway to ternary SNARE complex formation as summarized in the schematic diagram (Fig. 4*D*).

To quantitatively characterize this target SNARE intermediate, we measured both the affinity of syntaxin1 for SNAP-25 and the stoichiometry of this interaction. Determination of a dissociation constant (K_D) for a protein-protein interaction requires that saturable binding be achieved under equilibrium conditions. Therefore, we immobilized 1 pmol of SNAP-25 on Sepharose beads and incubated it with syntaxin1 in the concentration range of 10^{-9} to 10^{-5} M at 25 °C for 2 h. Bound syntaxin1 was measured by chemiluminescent immunoblotting in conjunction with a 12-bit cooled CCD camera. The binding was clearly saturable, and syntaxin1 bound to SNAP-25 with high affinity (K_D = 126 nM) (Fig. 5*A*). We used a quantitative Sypro Orange dye to analyze the stoichiometry of this interaction at a syntaxin concentration above the point of saturation. This fluorescent dye is a protein stain devoid of protein-to-protein variations (17). Acquisition of fluorescence on a cooled CCD camera within the linear dynamic range revealed that syntaxin1 bound to SNAP-25 in an ~1:1 molar ratio (Fig. 5*B*). Because the molar ratio of proteins in native SNARE complexes has not been quantified in a direct way, we purified the ternary complex from bovine brain cortex. The tight SNARE complex is disassembled by NSF ATPase (18) but can also be broken into its constituent parts by boiling (4). Boiling of the brain SNARE complex resulted in the appearance of monomeric syntaxin1, SNAP-25, and synaptobrevin2 on an SDS gel, and Sypro Orange quantitation yielded an approximately equimolar ratio for the three SNAREs (Fig. 5*C*). This direct measurement provides, for the first time, the protein ratio in the native SNARE complex composed of full-length proteins. The equimolar stoichiometry is mirrored by the x-ray analysis of the truncated ternary complex obtained using recombinant SNARE fragments (3) and, therefore, supports the physiological relevance of the crystal structure.

Our results so far suggested that the plasma membrane of chromaffin cells contains clusters of syntaxin1/SNAP-25 heterodimers. Botulinum toxin E (BoNT/E) potently blocks exocytosis by cleaving SNAP-25 before ternary SNARE complex formation (2). Although botulinum toxins can not cleave SNAREs in the ternary complex, their effect on the SNARE binary complex is unclear. The light chain of botulinum toxin E (BoNT/E LC) readily cleaved monomeric SNAP-25 (Fig. 6*A*), confirming the proteolytic activity of the toxin. We prepared a stoichiometric complex of syntaxin1 and SNAP-25 immobilized on anti-SNAP-25-Sepharose beads and then added BoNT/E LC. The toxin treatment led not only to cleavage of SNAP-25 but, strikingly, following standard washes, to full loss of syntaxin1 from the immobilized SNAP-25. This suggests that the loss of

A



B

Colocalisation of SNAP-25 with Syx in polar sections

Cluster	Positive (%)	Neutral (%)	Negative (%)	n
Syx/SNAP-25	95.24±1.80	4.75±1.80	0.10±0.10	310
SNAP-25/Syx	90.53±2.60	9.36±2.60	0.11±0.11	326

Analysis of SNAP-25 and Syx immunoreactive clusters

Cluster	Diameter (μm)	Area (μm^2)	Density (/μm^2)	n
SNAP-25	0.68±0.01	0.43±0.01	0.22±0.02	297
Syx	0.69±0.01	0.44±0.01	0.21±0.02	327

C

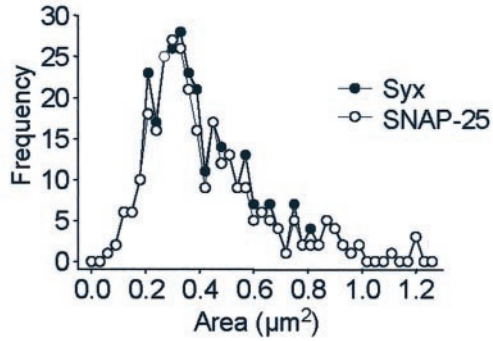


FIG. 2. **Quantification of syntaxin1 (*Syx*)/SNAP-25 clusters in chromaffin cells.** *A*, enlargement of double-immunostained polar confocal sections of fixed chromaffin cells. *Scale bar* represents 1 μm. *B*, image analysis of syntaxin1 (*Syx*) and SNAP-25 clusters. The degree of colocalization was measured by outlining clusters of high intensity for syntaxin1 from 10 polar confocal images, comparing them with SNAP-25 immunoreactivity, and scoring for positive (when the outlines completely overlap), neutral (when outlines partially overlap), and negative (when no overlap was detected). This was repeated using SNAP-25 immunostaining as a starting point. The mean diameter and area of the above mentioned outlines were calculated taking into account the point spread function of green and red fluorescence. The density was calculated as the number of clusters/μm^2 of plasma membrane imaged. *C*, frequency histogram of the area occupied by the syntaxin1 (*black circle*) and SNAP-25 (*open circle*) clusters showing a superimposable distribution.

the C-terminal 26 amino acids of SNAP-25 leads to disruption of the syntaxin1/SNAP-25 heterodimer (Fig. 6*B*). To confirm this finding, we immobilized the target SNARE heterodimer through GST-syntaxin1 on glutathione beads and tested again the effect of BoNT/E LC. Addition of BoNT/E LC completely disrupted the syntaxin1/SNAP-25 association, further demonstrating that the full-length SNAP-25 molecule is required for the stable interaction with syntaxin1 (Fig. 6*C*).

In light of the dramatic effect of BoNT/E LC on the stability of the syntaxin1/SNAP-25 heterodimer, we analyzed its impact on the target SNARE distribution in the plasma membrane of chromaffin cells. The addition of BoNT/E LC to permeabilized chromaffin cells led to marked changes in the localization of the target SNAREs. Following the toxin treatment, SNAP-25 immunoreactivity translocated partially into the cytosol in agreement with previous studies (19), whereas syntaxin1 immunoreactivity remained restricted to the plasma membrane (Fig. 6*D*). The polar confocal sections showed a dramatic disruption of normal syntaxin1/SNAP-25 colocalization, indicating that the association of target SNAREs in the plasma membrane is due to their high affinity interaction (Fig. 5*A*). Western immunoblotting of chromaffin cells confirmed that the majority of SNAP-25 had been digested by BoNT/E LC (Fig. 6*E*). Quantitative analysis of the SNAP-25/syntaxin1 distribution on the plasma membrane demonstrated a profound drop in colocalization of the two target SNAREs on the plasma membrane following botulinum toxin E treatment (Fig. 6, *F* and *G*).

DISCUSSION

The SNARE paradigm of intracellular membrane fusion, put forward a decade ago (20), has received overwhelming evidence through many independent lines of enquiry (1–3, 21). In particular, studies of the three neuronal SNARE proteins, synaptobrevin2 from secretory vesicles and the plasma membrane syntaxin1 and SNAP-25, contributed significantly to our understanding of the last step of vesicle exocytosis. These three proteins are also involved in exocytosis from many endocrine cells throughout the body, including adrenaline-secreting chromaffin cells and insulin-secreting pancreatic β-cells (13, 15). It is now generally accepted that the three SNARE proteins form a parallel four-helix bundle that can drive the fusion of two apposing membranes (3). However, the steps that actually lead to formation of the ternary SNARE complex are still poorly understood. By deciphering the pathway of SNARE assembly we will gain knowledge of how membrane fusion is regulated, because the availability of SNARE intermediates is likely to be an important point of control for the regulation of hormonal and neurotransmitter release.

We now provide four lines of evidence that syntaxin1 and SNAP-25 form stable heterodimers independent of synaptobre-

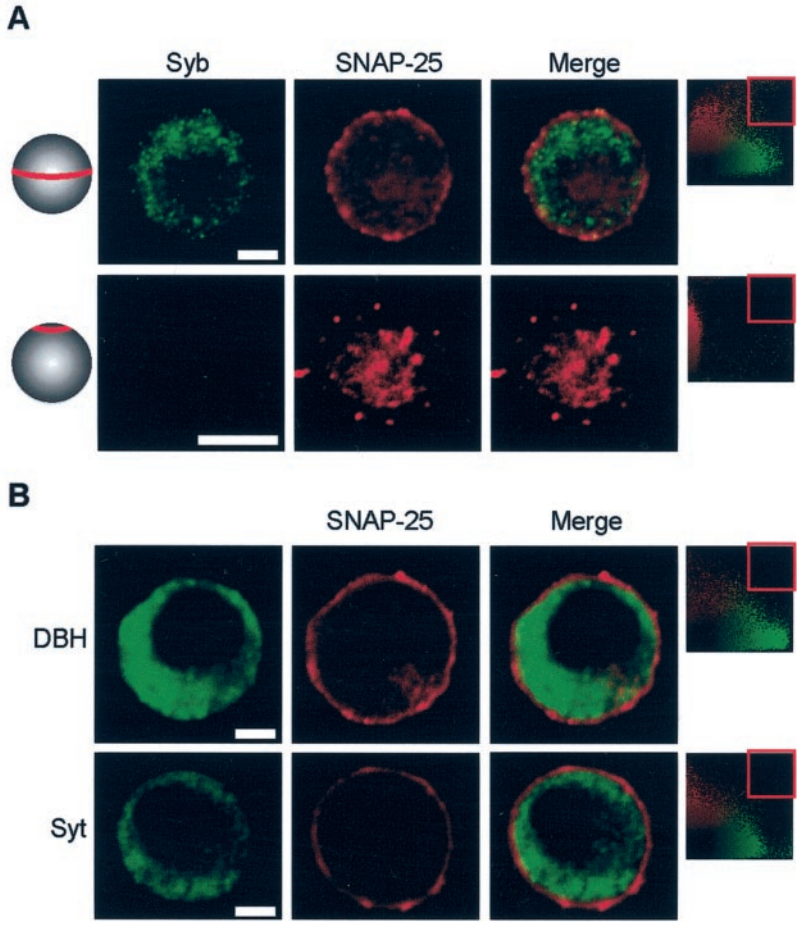


FIG. 3. **Distribution of vesicular markers in chromaffin cells.** *A*, synaptobrevin2 (*Syb*) distribution in chromaffin cells. An equatorial confocal section of chromaffin cell double-immunostained for synaptobrevin2 and SNAP-25. The scatter diagram of pixel intensity distribution reveals very little intermixing between red and green high intensity pixels, indicating the poor colocalization of the vesicular and target SNAREs. In a polar confocal section of the same cell, synaptobrevin immunoreactivity on the plasma membrane is absent when standard laser and photomultiplier tube settings are used. The scatter diagram of pixel intensity distribution reveals the absence of synaptobrevin immunostaining in the green channel. *B*, the vesicular markers deoxy-β-hydrolase (*DBH*) and synaptotagmin1 (*Syt*) do not colocalize with SNAP-25 in fixed chromaffin cells. The scatter diagrams of pixel intensity distribution show little intermixing between red and green high intensity pixels (*red window*).

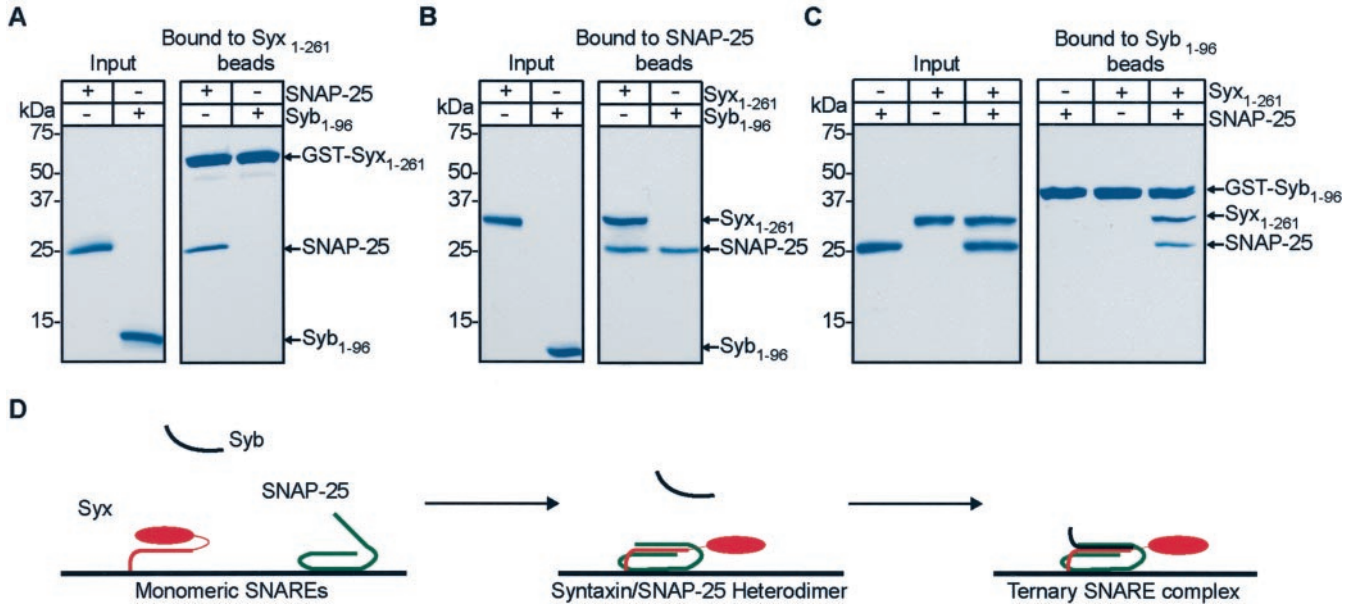


FIG. 4. **The syntaxin1 (*Syx*)/SNAP-25 heterodimer is the only stable binary SNARE complex.** The total input (*left panels*) and bound (*right panels*) proteins were analyzed by SDS-PAGE followed by Coomassie staining. *A*, GST-syntaxin1 ($GST\text{-}Syx_{1-261}$) attached to glutathione-Sepharose beads binds SNAP-25 and not synaptobrevin2 (Syb_{1-96}). *B*, SNAP-25 attached to anti-SNAP-25-Sepharose beads binds syntaxin1 (Syx_{1-261}) and not synaptobrevin2. *C*, GST-synaptobrevin2 ($GST\text{-}Syb_{1-96}$) attached to glutathione-Sepharose beads does not bind its monomeric partners but binds the syntaxin1/SNAP-25 heterodimer. *D*, schematic representation of the SNARE assembly pathway.

vin, both *in vitro* and *in vivo*. First, we showed that the two target SNAREs form the only stable intermediate on the pathway leading to the ternary SNARE complex (Fig. 4). Second, we demonstrated that syntaxin1 and SNAP-25 interact with high affinity and 1:1 stoichiometry (Fig. 5). Third, the distribution of the two target SNAREs coincide in clusters on the plasma membrane of chromaffin cells, whereas the majority of secretory granules are found at a distance from their target membrane in resting cells (Figs. 1–3). Fourth, botulinum toxin E can not only disrupt the syntaxin1/SNAP-25 heterodimer *in vitro*

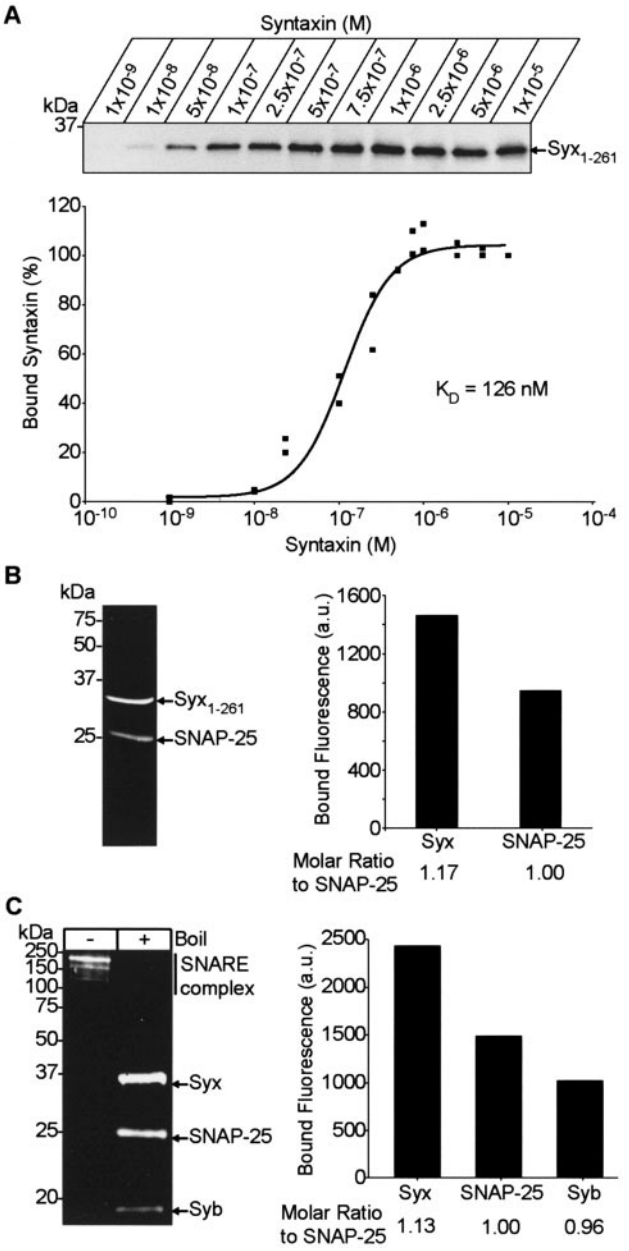


FIG. 5. **Syntaxin (*Syx*) interaction with SNAP-25 is of high affinity and equimolar stoichiometry.** *A*, calculation of the equilibrium dissociation constant for the syntaxin1 (Syx_{1-261})/SNAP-25 heterodimer. One picomole of SNAP-25, immobilized on Sepharose beads, was incubated in the presence of varying concentrations of syntaxin, and bound material was analyzed by Western immunoblotting using an anti-syntaxin antibody. Quantification of bound syntaxin, using a cooled 12-bit CCD camera, yielded a dissociation constant (K_D) of 126 nM. *B*, stoichiometry of the syntaxin1/SNAP-25 heterodimer. SNAP-25, immobilized on Sepharose beads, was incubated with a saturating concentration of Syx_{1-261}. Bound protein was analyzed by SDS-PAGE and Sypro Orange staining. Quantification of bound fluorescence using a cooled 12-bit CCD camera yields a molar ratio of ~1:1. *C*, stoichiometry of the native ternary SNARE complex. The SNARE complex, purified from bovine brain, was disrupted by boiling in the sample buffer. Staining with Sypro Orange and quantitation of bound fluorescence gives a molar ratio of ~1:1:1 for syntaxin1, SNAP-25, and synaptobrevin2, which have molecular masses of 35, 25, and 18 kDa, respectively. *a.u.*, arbitrary units.

but also disorders the target SNARE clusters in chromaffin cells (Fig. 6). Taken together, our data show that, in chromaffin cells, syntaxin1 and SNAP-25 form the sole stable SNARE intermediate, which exists on the plasma membrane in discreet clusters.

The demonstration of a single high affinity binary complex between syntaxin1 and SNAP-25 defines unambiguously the SNARE assembly pathway. Our data agree with biophysical measurements that proposed the syntaxin1/SNAP-25 heterodimer as a possible intermediate (22). Using chemiluminescent immunoblotting, some interactions between SNAP-25 and synaptobrevin were observed previously (4, 23). However, these interactions have not been investigated in a quantitative manner and could well be sub-stoichiometric, because large amounts of protein were immobilized to detect binding. Our direct protein quantitation in the syntaxin1/SNAP-25 heterodimer revealed a 1:1 molar ratio (Fig. 5*B*). Significantly, the native SNARE complex also possesses equimolar protein ratios (Fig. 5*C*) in agreement with the crystal structure of the recombinant truncated complex (3). This validates independently the use of Sypro Orange as a quantitative protein dye (17) and calls into question the previous indirect estimation of two syntaxin molecules with one molecule of SNAP-25 in a binary complex (24). Based on our protein quantification of SNARE complexes, we suggest that transition from the syntaxin1/SNAP-25 heterodimer to the ternary SNARE complex simply requires engagement of synaptobrevin.

The localization of syntaxin1 and SNAP-25 on the chromaffin cell plasma membrane, in the absence of synaptobrevin, agrees well with the SNARE distribution in neurons and pancreatic secretory cells (15, 25). The segregation of secretory granules from the plasma membrane, in chromaffin cells, is thought to be due to the cortical actin network (13, 26). The SNARE organization observed here is in contrast to a study of PC12 membrane sheets, which concluded that the plasma membrane contains synaptobrevin in large amounts and that the SNAREs do not interact in resting cells (8). It is possible that the brief sonication of PC12 cells used to prepare plasma membrane sheets can lead to mixing of subcellular compartments and redistribution of SNAREs. Furthermore, some PC12 cell lines, being tumor cells, may have lost their SNARE organization, whereas chromaffin cells, as a primary culture, retain the highly organized secretory machinery. Nevertheless, partial colocalization of syntaxin and SNAP-25 has been observed in intact PC12 cells upon the expression of exogenous fluorescent syntaxin (14).

The small size of syntaxin1/SNAP-25 clusters (700 nm in diameter) suggests that vesicles are either actively targeted to the fusion sites or, instead, the random collision of vesicles with these clusters leads to exocytosis. Significantly, recent dynamic single vesicle imaging studies revealed that only a proportion of approaching vesicles fuse with the plasma membrane (27, 28). Chow and co-workers observed defined sites of repeated vesicle docking and fusion in chromaffin cells (29) with a spatial distribution similar to the syntaxin/SNAP-25 clusters (Fig. 1*B*). Although our approach identified the target SNARE clusters in fixed chromaffin cells, this method has its own limitations, as it does not allow investigation of the dynamics of vesicle movement and fusion with the plasma membrane. Alternative methodologies such as genetic replacement of the endogenous target SNAREs with fluorescent syntaxin1 and SNAP-25, labeling of secretory vesicles, and the use of total internal reflection fluorescence microscopy of living cells will be useful for further advances. Our finding of the well organized target SNARE machinery in resting chromaffin cells is in agreement with the characterization of the syntaxin1/SNAP-25 heterodimer as the only stable intermediate in the SNARE assembly pathway and provides a clearer picture of steps underlying SNARE-mediated exocytosis.

The discoveries that botulinum toxins cleave monomeric SNAREs and block neuronal exocytosis have provided an important cornerstone for the SNARE paradigm (2, 5). We showed

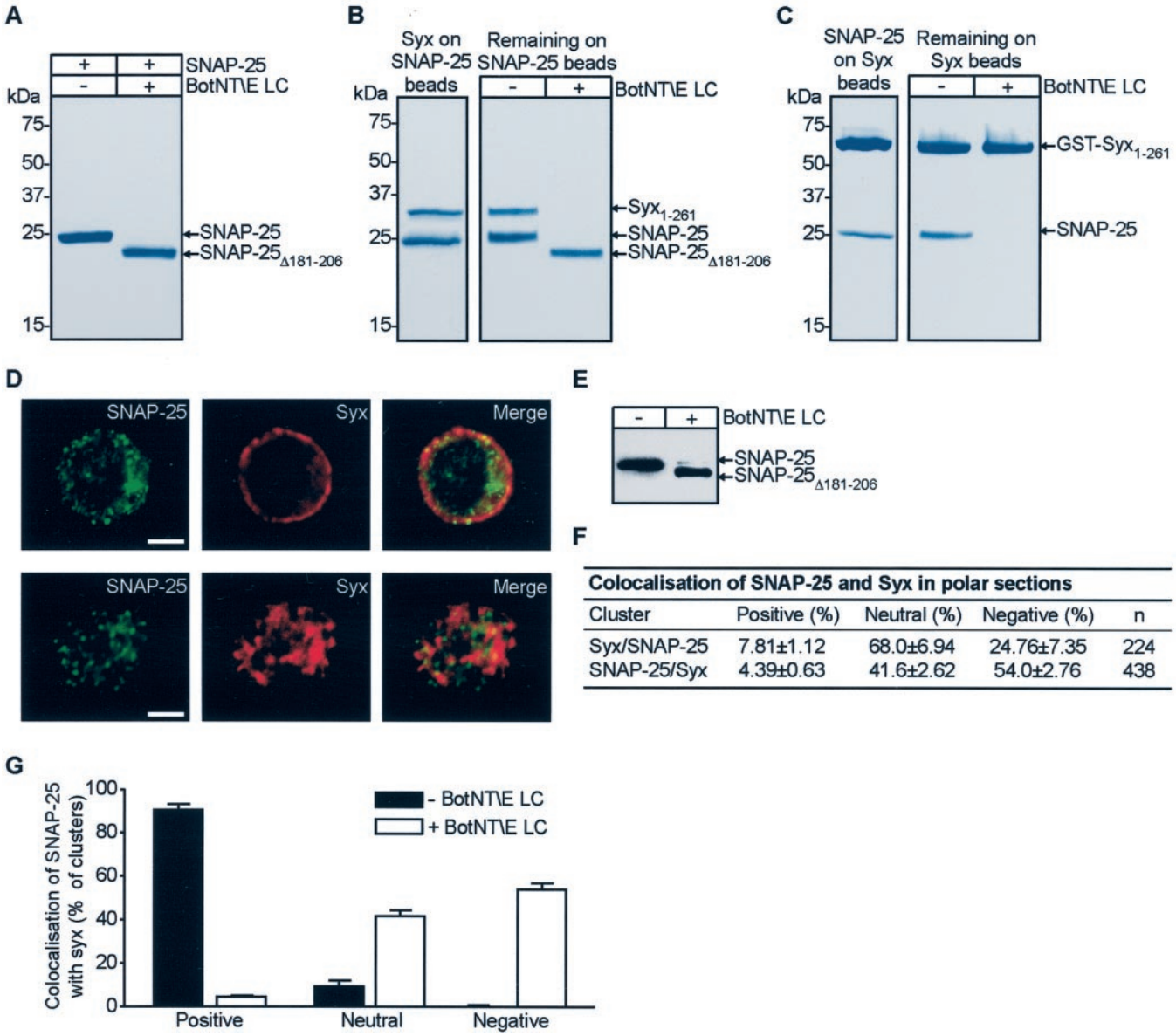


FIG. 6. **Cleavage of SNAP-25 by the light chain of botulinum toxin E results in disruption of the syntaxin1/SNAP-25 heterodimer.** *A*, SNAP-25 is readily cleaved by BoNT/E LC *in vitro*. Coomassie-stained gel is shown. *B*, SNAP-25 is cleaved by BoNT/E LC even when in the syntaxin1/SNAP-25 heterodimer. The target SNARE heterodimer was preassembled on anti-SNAP-25-Sepharose beads and incubated with or without BoNT/E LC for 1 h at 37 °C. Beads were washed, and the protein remaining on the beads was analyzed by SDS-PAGE and Coomassie staining. Note that the syntaxin1 (Syx_{1-261})/SNAP-25 heterodimer does not dissociate upon long incubation in the absence of BoNT/E LC. *C*, BoNT/E LC cleavage of SNAP-25 disrupts the syntaxin1/SNAP-25 heterodimer. GST-synaxtin1 ($GST\text{-}Syx_{1-261}$) attached to Sepharose beads was incubated with SNAP-25, and unbound SNAP-25 was removed by washing. The subsequent cleavage with BoNT/E LC results in a complete loss of SNAP-25 from the syntaxin beads. Coomassie stained gels are shown. *D*, BoNT/E LC disrupts the normal colocalization of syntaxin1 (*Syx*) and SNAP-25. Equatorial and polar confocal sections of a double-immunostained chromaffin cell. *E*, Western immunoblotting of chromaffin cells confirms cleavage of SNAP-25 by BoNT/E LC. *F*, BoNT/E LC treatment has a deleterious effect on the colocalization of syntaxin1 and SNAP-25. The degree of colocalization was measured as in Fig. 2. *G*, summary of SNAP-25 colocalization with syntaxin1 (*syx*) in control cells and after their treatment with BoNT/E LC. The toxin treatment results in a sharp drop in positive colocalization with a parallel increase in neutral and negative signals.

that SNAP-25, even when associated with syntaxin1, can also be targeted by botulinum toxin E leading to SNAP-25 cleavage and disruption of the binary complex. Because the target SNARE clusters likely define sites for ternary SNARE complex formation, disruption of these clusters by the toxin may contribute to the well established blockade of exocytosis (6, 7). The fact that the majority of SNAP-25 is in association with syntaxin on the plasma membrane of chromaffin cells also indicates that botulinum toxin E must target this heterodimer to block exocytosis.

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High Affinity Interaction of Syntaxin and SNAP-25 on the Plasma Membrane Is Abolished by Botulinum Toxin E

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